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Concentrations of Abscisic Acid and Indole-3-Acetic Acid in Soybean Seeds during Development[1]

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ABSTRACT

Concentrations of abscisic acid (ABA) and indole-3-acetic acid (IAA) in seed parts were determined during reproductive development of soybean plants (*Glycine max* [L.] Merr. cv 'Chippewa 64'). The concentration of ABA and IAA changed independently in individual seed parts with time. Measurement of the level of ABA and IAA in whole seeds masked the changes which occurred in individual seed tissues. The concentration of ABA was generally highest and that of IAA was generally lowest in the embryonic axis of soybean seeds. In the testa, the IAA concentration was generally highest while the ABA concentration was generally the lowest compared to other parts of the seed.

The endogenous plant growth substances ABA and IAA have been implicated in the correlative control of many plant developmental processes (1, 9-13, 15, 17). Both compounds have been identified in reproductive structures of soybeans (2, 6). Little information is available, however, concerning the distribution of these compounds in different seed parts during seed development.

This report describes the changes in concentration of both ABA and IAA in seed tissue of soybean during reproductive development. Both compounds were present in sufficient quantities to permit detection in cotyledons, embryonic axes, and testae of individual seeds from the earliest date on which seeds could easily be dissected.

MATERIALS AND METHODS

Plants. Soybean plants (*Glycine max* [L.] Merr cv 'Chippewa 64') were grown in a growth chamber as previously described (12). Plants were sampled at 1-week intervals from 1 week prior to midflowering until 90 d postplanting. Planting of soybeans was staggered in order to optimize use of growth chamber space. Three replicates of each treatment for plants of each age were sampled in a completely random design. The experiment was repeated, and since there was no difference between measured parameters in the two experiments for plants of any age, data from both sampling dates were pooled to give six replicates per treatment per plant age. All data presented represent the mean of six replicate measurements on individual plants unless otherwise indicated.

Pods from the axil of the second most recently expanded trifoliolate leaf were sealed in tared plastic bags and immediately placed on dry ice. Pod weights were determined before storing pods (−20°C) for further analysis.

Extraction. The single largest seed from the sampled node was dissected into the testa, cotyledons, and embryonic axis. Each seed part was extracted in 200 μl of cold (−80°C) 80% aqueous methanol containing butylated hydroxytoluene (10 mg/l, Calbiochem), citric acid (500 mg/l, Spectrum, Redondo Beach, CA), and internal standards of 500 dpm each of radiolabeled IAA and ABA (51.5 mCi/mmol 1-[^{14}C]IAA, 11.3 mCi/mmol 2-[^{14}C]ABA, Amersham) in a 1.5-ml polyethylene centrifuge tube. Seed parts were pulverized in the centrifuge tube with a glass pestle. After extraction for 4½ h at 4°C, the homogenates were centrifuged at 12,000*g* for 15 min (Minifuge 12, Beckman, Fullerton, CA). The supernatant was decanted and the pellet resuspended in extraction medium without internal standards. After extraction for 2 h at 4°C the samples were centrifuged as before and the supernatants combined. These combined extracts were reduced to the aqueous phase in silanized glass tubes under reduced pressure at 30°C and frozen (−20°C) until further analysis.

Separations. Aqueous seed extracts were thawed, brought to a volume of 1.0 ml with deionized-distilled H_2O, and filtered (1.0 μm cellulose nitrate, Microfiltration Systems, Dublin, CA) prior to preparative HPLC. Filtered samples were placed in 6-ml tuberculin syringes and injected into the holding loop of a six-port high pressure injection valve (HP7000, Valco, Houston, TX) with a syringe autosampler (designed in the investigator's laboratory). Samples were eluted from a preparative PRP-1 column (10 μm particle diameter, Hamilton, Reno, NV, 10 mm × 10 cm column) with a 30 min gradient from 0.01 N NaH_2PO_4 in 10% ethanol to 0.1 N Na_2HPO_4 in 50% ethanol at 2.0 ml/min. The fraction in which authentic standards of IAA and ABA eluted from the PRP-1 column was diverted to a preparative C_{18} column (7 μm particle diameter, Nucleosil, Macherey-Nagel, 10 mm × 15 cm column) with a high pressure valve (model 7010, Rheodyne, Cotati, CA). IAA and ABA were eluted from the C_{18} column with a 30-min linear gradient from 0.1 N acetic acid (pH 2.8) to 0.1 N acetic acid in 50% ethanol at 2.5 ml/min. Fractions containing ABA and IAA were collected at the elution times corresponding to the elution times of authentic standards of these compounds. Sample injection, solvent delivery (Waters Associates M6000A, Milford, MA), and fraction collection (ISCO 568, Lincoln, NE) were automated with a laboratory controller (SLIC 1400, Systec Inc., Minneapolis, MN). Fractions containing ABA were dried under reduced pressure (30°C) and stored for analysis by GLC. Fractions containing IAA were reduced to 1.0 ml of

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aqueous eluant under reduced pressure (30°C) and frozen (−20°C).

A second preparative liquid chromatographic system was necessary to remove impurities from the IAA fraction prior to quantitative analysis. IAA fractions (1.0 ml) from the initial preparative separation were thawed, filtered as before, and loaded into 6-ml tuberculin syringes. Samples were displaced from syringes into the holding loop of a high pressure six-port injection valve (Rheodyne 7124) with a syringe autosampler. Samples were injected onto a C_{18} column (7 μm particle diameter Nucleosil, 4.2 mm × 15 cm column) in 0.2 N acetic acid and eluted at 1.0 ml/min. The mobile phase was changed to 10/20/70 acetonitrile/ethanol/water in 0.2 N acetic acid with a 3-min gradient. The IAA fraction was collected into silanized glass tubes at the retention time of authentic IAA, neutralized with 0.1 N NaOH and dried under reduced pressure at room temperature. Injection, solvent delivery (Waters M6000) for fraction collection (LKB 7000, Rockville, MD) were controlled with a microprocessor controller (SLIC 1400). Dried IAA fractions were stored frozen until further analysis.

Analysis. ABA was methylated with diazomethane (6), transferred to 1.5 ml or 250 μl GC autosampler vials, and dried under N_2. Samples containing ABA methyl ester were dissolved in ethyl acetate and chromatographed on a capillary gas chromatograph (model 5880A, model 7671A autosampler, Hewlett Packard, Avondale, PA). One μl of sample was injected into a quartz inlet liner packed with 3% SP2100 on Supelcoport 100/120 (Supelco, Bellefonte, PA), split ratio 15:1, and chromatographed isothermally (165°C) on a 0.32 mm × 30 m fused silica capillary column (SE-30 coated or DB-1, J&W Scientific, Rancho Cordova, CA). The carrier gas was H_2 with a linear velocity of 50 cm/s (N = 85,000 for methyl abscisate at $k' = 12.5$). Argon/methane (95/5) was added as makeup gas at 100 cc/min at the inlet of the electron capture detector (^{63}Ni). ABA in plant samples was quantitated by external standardization to authentic methyl abscisate standards using peak height measurement. Losses of ABA during extraction and purification were accounted for by measuring isotope dilution of the radiolabeled internal standards (3). Recovery of internal standard was determined by dissolving the methylated samples remaining in the autosampler vial in scintillation cocktail (Aquasol 2, New England Nuclear) and counting the recovered radioactivity with a scintillation spectrophotometer (LS 9000, Beckman).

Frozen IAA samples from the second preparative chromatographic step were dissolved in 60 μl of 0.02 N citrate buffer (pH 6.2). Samples were injected (50 μl, WISP 710 B, Waters Associates) onto a strong anion exchange column (7 μm particle diameter, Zorbax SAX, Dupont, Wilmington, DE, 4.2 mm × 15 cm column) and eluted with a mobile phase of 0.02 N citrate, 0.05 N Na_2HPO_4, 0.02 N EDTA in 10% aqueous methanol. The column eluant was first passed through a fluorescence monitor (FS 950, Kratos, Westwood, NJ; excitation 254 nm, emission 340 nm) then through an oxidative amperometric detector (14) (LC_2A, Bioanalytical Systems, West Lafayette, IN; +0.95 volt *versus* Ag/AgCl). IAA was quantitated by measuring the peak height for the fluorescence response to IAA (Waters Associates Data Module) and comparing to an external standard curve for response to authentic IAA. The ratio of responses of the two detectors to putative IAA peaks in plant samples was compared to the detectors' response ratio for authentic IAA standards. If the detectors' response ratio did not fall within one standard deviation of the mean ratio determined from the analysis of 10 standards, the peak was deemed impure, and not used for quantitation. Losses of IAA were determined as for ABA by collecting the HPLC effluent at the time of elution of IAA and determining the isotope dilution of the internal standard (3). Recovery of IAA and ABA from plant samples was 51 ± 10% and 74 ± 6%, respectively.

RESULTS

Seed Growth. First flowers appeared on soybean plants between 40 and 42 d after planting when plants had seven fully expanded trifoliolate leaves. Flowering continued until 54 d after planting. Seed fresh weight was determined for the largest seed at the node of the second most recently expanded trifoliolate leaf at each sampling time (Fig. 1). Seed growth was slow during the first 2 weeks after anthesis, during which time the pods elongated to about 90% of maximum length. By this time seeds could be dissected into individual parts (cotyledons, testa, and embryonic axis). There was little evidence of endosperm on the earliest sampling day on which seeds could be dissected into individual parts, but the cotyledons and embryonic axis may have been contaminated by the small amount of endosperm which can persist until seed maturity (5).

Rapid seed filling began in the 3rd week after fertilization and continued for 2 weeks, after which time the seeds began to dry. The time course of fresh weight increase (Fig. 1) during the first 3 weeks of sampling shows a slower rate of fresh weight increase than would be observed if seeds at one node had been followed throughout development, since seeds sampled on days 45 and 59 were fertilized on different days. After day 59, sampled seeds all had been fertilized within 24 h of each other.

Early in seed development the testa and cotyledons weighed about the same (Fig. 1). By day 66 the testa accounted for less than 20% of the seed fresh weight. This percentage decreased as the cotyledons filled and the weight of the testa declined as the

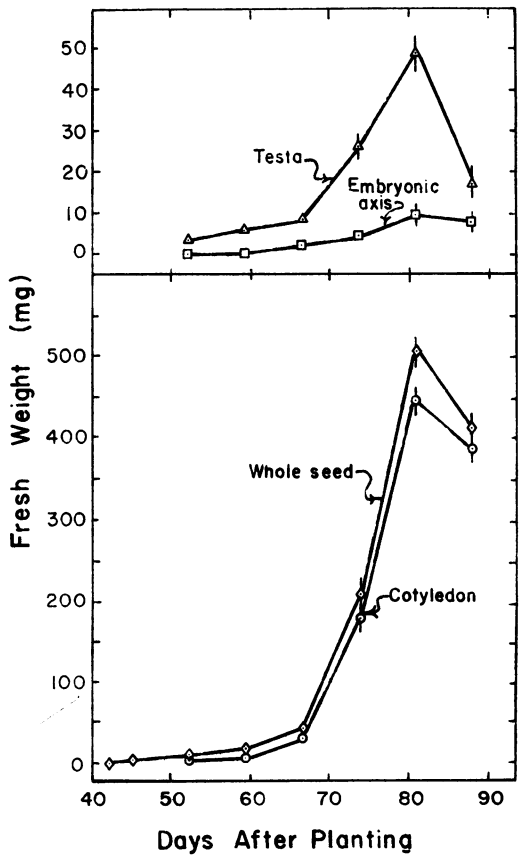

FIG. 1. Fresh weight of largest seed at the node of the second most recently expanded trifoliolate leaf. A, Weight of the testa and embryonic axis; B, weight of the whole seed and cotyledon. Means of six replicates and their SE are shown.

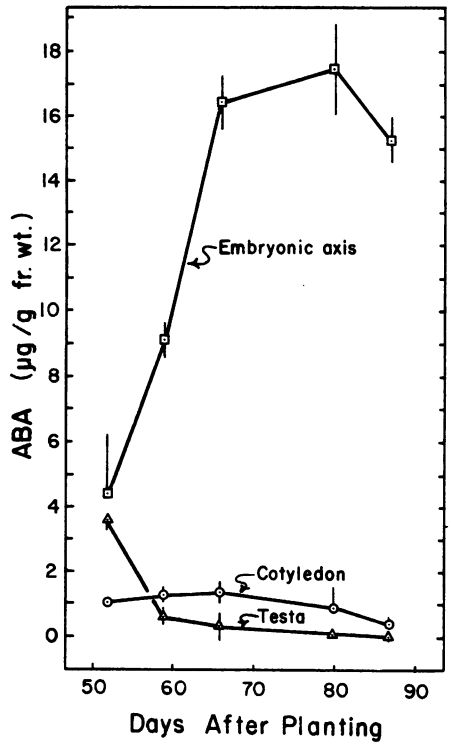

FIG. 2. Concentration of ABA in parts of seeds at node of second most recently expanded trifoliolate leaf. Means of six replicates and their SE are shown.

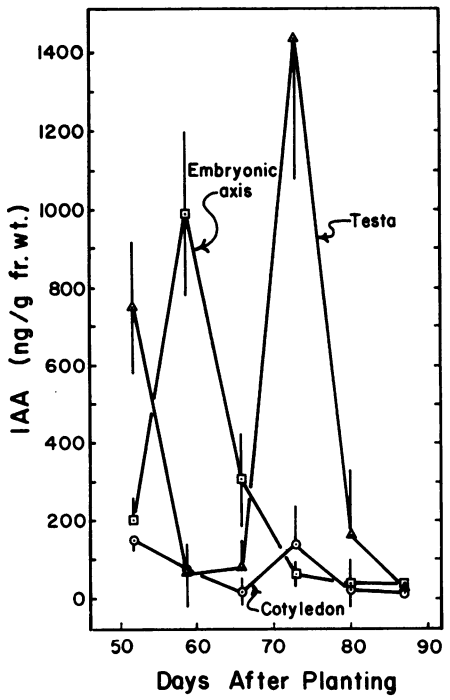

FIG. 3. Concentration of IAA in parts of seeds at node of second most recently expanded trifoliolate leaf. Means of six replicates and their SE are shown.

seed approached maturity. The embryonic axis accounted for a small percentage of the seed fresh weight throughout seed development.

IAA and ABA Levels. Sampling for IAA and ABA in individual seed components began on day 52 when seed could first be dissected. On day 52, the embryonic axis and the testa contained about equal concentrations of ABA, while the concentrations in the cotyledons was less (Fig. 2). During development, the ABA concentration of the embryonic axis increased, reaching a maximum between days 66 and 80 and then declining slightly as seeds matured. The ABA concentration in the testa declined after day 52, was very low during rapid seed fill, and was not detectable on the final sampling date (Fig. 2). On day 52 the ABA concentration in the cotyledons was relatively low and remained unchanged until the last sampling day when it declined (Fig. 2).

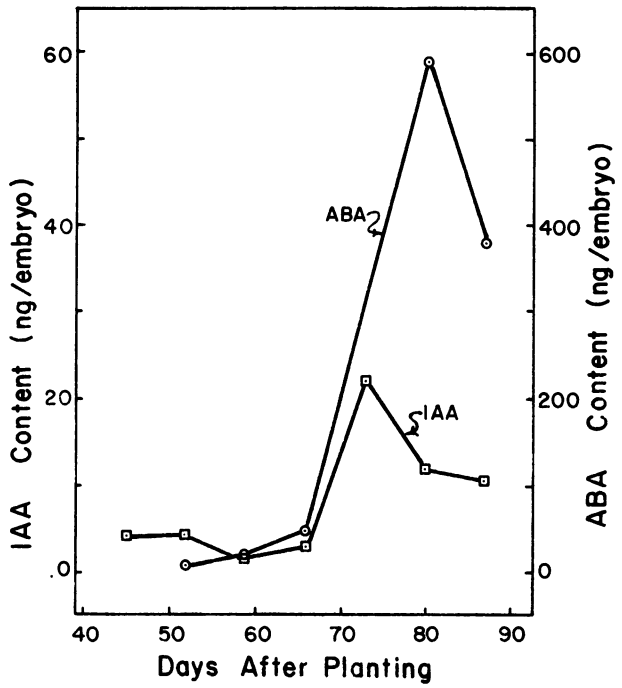

FIG. 4. Amount of ABA and IAA in the embryo of the largest seed at the node of the second most recently expanded trifoliolate leaf. Means of six replicates are derived from the sum of the seed component data presented in Figures 2 and 3.

Concentrations of IAA in seed parts of soybean were dynamic in this study (Fig. 3) and in general were higher than levels reported previously (3, 11). Accumulation of IAA in the seed (Fig. 4) followed a similar profile to that of ABA with the exception that the maximum content of IAA in seeds or embryos occurred prior to the maximum in ABA content. When IAA was at its highest level in the seed (day 73), the highest concentration of the IAA occurred in the testa rather than in the cotyledon, as was the case for ABA. The concentration of IAA in the embryonic axis was highest when the pod was increasing in length at its most rapid rate.

DISCUSSION

Levels of ABA found in seeds have been reported on a basis of mass of ABA per unit of seed or embryo weight and on a basis of ABA per single seed or embryo (1, 6, 9, 11). Expressing ABA levels in seeds by either of these methods infers a uniform distribution of ABA in the heterogeneous tissue and cell types which make up the seed. Each of these tissues and cell types have different functions in embryogenesis and seed development. Since these functions are likely to be affected differently by ABA, the importance of ABA concentration *per se* is likely to be time and tissue dependent.

In general, it has been observed that the amount of ABA in seeds increases in parallel with seed weight (Fig. 4) (9, 11). On closer examination, this observation masks changes in the con-

centration of ABA in individual seed parts (Fig. 2). Previously in soybean, the concentration and mass of ABA has been shown to differ in the testa, cotyledon, and embryonic axis during rapid seed filling (11). In this study, during the time when the amount of ABA in the seeds was increasing rapidly (days 66 to 80, Fig. 4), the ABA concentration in the cotyledons was not changing. The increase in total mass of ABA in the seed was accounted for by the rapid increase in mass of the cotyledons despite the decreasing ABA concentration in that tissue.

A number of possible functions for ABA in developing seeds have been postulated. The suggested roles include control of phloem unloading (16), suppression of precocious germination (2, 11, 17), and promotion of storage protein accumulation (1, 8). No causal relationship has been demonstrated *in vivo* for endogenous ABA in these processes. This is in part due to the gross simplification of biological function imparted by measuring hormone levels in heterogeneous tissues (or entire organisms in the case of whole embryos) and in part due to lack of understanding of the correlated processes.

It has been demonstrated that the concentration of ABA in individual seed tissues can be determined through much of embryogenesis and development. This information coupled with the ability to assay specific events in seed development, like transcription and translation of specific storage protein messenger RNA (8), will allow better assessment of the *in situ* role of ABA in seed tissue development.

Less information is available concerning the occurrence of IAA than of ABA in legume seeds. The presence of IAA (3, 7, 14) and conjugates (3, 7) of IAA has been demonstrated in soybeans, but the amounts of IAA in individual seed parts throughout embryogenesis have not been previously reported. In previous studies it has been shown that IAA is present in whole immature soybean seeds at levels of 50 to 200 ng/g fresh weight (14) and in mature seeds at a level of only 4 ng/g (3). While, in this study, the IAA concentration in the whole seed would have been within this range at several stages of development, the levels in individual seed parts were often much higher (as much as 1400 ng/g fresh weight, Fig. 3). In instances when the concentration of IAA in the embryonic axis or testa was relatively high, an analysis of IAA in the whole seed would have resulted in a lower reported value due to the low concentration of IAA in the cotyledons (Fig. 3) which contributed the bulk of the seed weight (Fig. 1).

IAA has been suggested as a correlative signal from seeds involved in control of development of other organs (9, 10, 15). Few studies have provided evidence for the involvement of IAA in seed development. However, exogenously applied auxin has been shown to be transported to other parts of the plant (4). The testa is the transport interface between the developing embryo and other maternal tissues, since there are no symplastic connections between the two. It is notable then, that the concentration of IAA in the testa was relatively high at all sampling times during this experiment. Whether or not IAA in the testa serves as a pool for transport to other tissues of the embryo or maternal plant will be addressed in a subsequent investigation.

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